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Increased understanding of the dry season survival mechanisms of *Anopheles gambiae* (*An. gambiae*) in semi-arid regions could benefit vector control efforts by identifying weak links in the transmission cycle of malaria. In this study we examine effect of seasonal indicators on morphologic and chemical characteristics known to contribute to suppression of water loss in mosquitoes. *An. gambiae* body size (indexed by wing length), mesothoracic spiracular index ((spiracle-length/wing-length)*100), and cuticular hydrocarbon (CHC) profiles were examined for their ability to differentiate mosquitoes exposed to aestivating and non-aestivating conditions in a laboratory setting. Mosquitoes exposed to aestivating conditions exhibited larger wing lengths, larger mesothoracic spiracular indices, and greater total CHC amount (standardized) than mosquitoes exposed to non-aestivating conditions. Total CHC amount increased with both mating and age. Mean n-alkane retention time (a measure of mean n-alkane chain-length) was lower in mosquitoes exposed to aestivating conditions, and increased with age. Individual CHC peaks were examined, and several CHCs were identified as potential biomarkers of aestivation, age, and insemination status. This study indicates that aestivation status of nulliparous female *An. gambiae* can be determined using both morphologic and chemical biomarkers.

Keywords: *Anopheles gambiae,* aestivation, cuticular hydrocarbon, spiracle, wing length

IDENTIFICATION OF MORPHOLOGIC AND CHEMICAL MARKERS OF AESTIVATION CONDITIONS IN FEMALE *ANOPHELES GAMBIAE* MOSQUITOES

by

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_____
Committee Chair

This thesis is dedicated to Ron Rivera who, before being brought down by the bite of a mosquito, raised so much awareness, and saved so many lives from the dangers of waterborne disease. RON PRESENTE!

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

Purpose.

Malaria is a disease caused by infection from any of four human parasites in the genus *Plasmodium*, and is spread through the bite of infected female Anopheline mosquitoes (Najera and Hempel, 1996). In 2008, there were an estimated 243 million cases of malaria worldwide, the vast majority (85%) of which were in Africa (WHO, 2009). Insecticide-treated nets and indoor residual insecticide spraying are the two most powerful and most broadly applied mosquito vector control interventions, intended to prevent infective bites and reduce malaria transmission at the community level (WHO, 2009). However increasing resistance of mosquito vectors to insecticides, especially pyrethroids, presents an urgent threat to worldwide vector control efforts (WHO, 2009). Despite decades of intervention, malaria remains a primary international health concern, responsible for approximately one million deaths per year (Aregawi et al., 2008; Gray and Bradley, 2005).

Regions of malaria transmission can be defined as either perennial, where conditions are always suitable for transmission, or seasonal, where conditions are only suitable for transmission during certain times of the year (Craig et al., 1999). In the latter, vector reproductive period and disease transmission fluctuate considerably with seasonal

variation in temperature, precipitation, and day length. During unfavorable (dry) weather, spatial distribution of vectors tends to contract (Reisen, 2010). Since village-scale clustering of adult mosquitoes is more evident during population lows (Ribeiro et al., 1996), the substantial seasonality of mosquitoes outside of permanently humid areas provides a good opportunity for vector control intervention.

Lack of rainfall is especially restrictive to reproduction of *Anopheles gambiae (An. gambiae)*, which requires surface water for larval development (Beier et al., 1990; Koenraadt et al., 2003; Lehmann et al., 2010). In the Sahel region of Mali in western Africa, malaria vectors include *An. gambiae* and *An. arabiensis* (Huestis et al., In Press; Lehmann et al., 2010). During the dry season, when surface water needed for larval development dries up for four to eight months of the year, *An. gambiae* can be found in small numbers, while An. arabiensis seems to virtually disappear (Lehmann et al., 2010). After the onset of rains, the population size of *An. gambiae* increases as much as ten-fold within seven days. However embryonic and larval development of Anopheles require at least nine days, indicating that the adults were present before the rains began (Lehmann et al., 2010) and that the population increase is not a result of emergence from newly laid or desiccation-resistant eggs. A study in Sudan found that ovarian development occurs very slowly in female *An. gambiae* collected during the dry season. These females undergo only one gonotrophic cycle during the entire dry season, resulting in gravid females that are ready to deposit eggs immediately after the onset of rains (Omer and Cloudsley-Thompson, 1968). This adaptation is suggestive of aestivation, a dormant state

associated with extended longevity of adult females in the dry season (Holstein, 1954; Omer and Cloudsley-Thompson, 1968). Aestivation is considered an adaptation for survival during hot, dry periods, and has been observed in many insects, including the winter mosquito, *Culiseta inornata* (Barnard and Mulla, 1977), the Hessian fly, *Mayetiola destructor* (Benoit et al., 2010), the tiger moth, *Cymbalophora pudica* (Kostal et al., 1998), and the alfalfa weevil, *Hypera postica* (Tombes, 1964).

While several studies have reported low-density presence of female Anopheline mosquitoes during the late dry season (Charlwood et al., 2000; Jawara et al., 2008; Omer and Cloudsley-Thompson, 1968, 1970), there is little hard evidence that rapid emergence of malaria vectors immediately after onset of rains is a result of extended life by aestivation (Lehmann et al., 2010; Omer and Cloudsley-Thompson, 1970). The recapture of one marked female *An. gambiae* in May of 2009, 7 months after her release, confirmed that female aestivation does occur in Mali (Lehmann et al., 2010). The extent to which adult aestivation contributes to the buildup of *An. gambiae* populations in the wet season in Mali was investigated by comparing wet season populations of two control sites (monitored only) to that of two sites receiving weekly dry-season pyrethrum sprays (Adamou et al., In Press). *An. gambiae* wet-season density was reported to be 30% lower in the two sites that received pyrethrum treatments, while density of *An. arabiensis*, did not differ significantly. These findings suggest that in Mali, migration may contribute to dry-season survival of *An. arabiensis*, while adult aestivation likely plays a significant role in *An. gambiae* dry-season survival (Adamou et al., In Press; Huestis et al., In Press).

Although aestivation is associated with warm-dry weather, cold-dry weather can also induce a dormant state of extended adult female longevity, termed winter diapause. Though evidence of winter diapause has been observed in many temperate mosquito species (Anderson, 1968; Benoit and Denlinger, 2007), the physical and chemical adaptations associated with aestivation have not yet been well documented in *An. gambiae* (Adamou et al., In Press; Gray and Bradley, 2005; Lehmann et al., 2010). Suppression of water loss is a primary characteristic of species that face weather-induced desiccation and is expected to be important for the survival of *An. gambiae* during the dry season in Mali. In winter-diapausing *Culex pipiens*, three features were found to contribute to suppression of water loss: larger body size, reduced metabolic rate (and consequent reduction in respiration), and increased quantity of cuticular hydrocarbons (see definition below) (Benoit and Denlinger, 2007). Increased understanding of the morphological and chemical characteristics that distinguish aestivating *An. gambiae* populations could contribute significantly to the development of novel strategies for vector control targeted at dry-season survivors (Minakawa et al., 2001). This is particularly important in the face of increasing insecticide resistance.

Morphologic Markers.

Body Size.

Cuticular transpiration is one mechanism through which insects loose water (transcuticular water loss) (Zachariassen et al., 1987). Increased body size enables suppression of transcuticular water loss through a decrease in the body's surface area to

volume ratio (Benoit and Denlinger, 2007; Day and Lawrence, 2000). In mosquitoes, wing length and body mass are considered reliable indices of body size (Ameneshewa, 1996; Kaufmann and Briegel, 2004; Lounibos et al., 1995; Weber, 1990). *An. gambiae* adult wing length and dry mass were found to increase as temperature increased from 24º to 27ºC, when mosquitoes were reared at low densities (Lyimo et al., 1992). In *An. quadrimaculatus,* individuals reared under a short photoperiod were larger in both wing length and dry body weight than individuals reared under a long photoperiod (Lanciani, 1992). In *Culex pipiens*, initial and dry body mass were found to be higher in adult females collected during the winter (diapausing) than in females collected in the summer (Benoit and Denlinger, 2007). Wing length has been found to be positively correlated with survival and fecundity in adult female mosquitoes (Ameneshewa, 1996; Day et al., 1990; Lyimo and Takken, 1993). For example, in adult female *An. arabiensis*, wing length was found to be positively correlated with probability of survival, likelihood of insemination, and overall number of egg batches produced (Ameneshewa, 1996). In *Culex nigripalpus,* large females were more successful at acquiring blood meals and developing eggs during periods of drought (Day et al., 1990). If aestivating *An. gambiae* are larger than non-aestivators, the positive correlation between wing length and fecundity/survival suggests that aestivating mosquitoes could play an important role in the perpetuation of isolated or semi-isolated mosquito populations.

Spiracle Size.

Reduced metabolic rate may also contribute to mosquito dry-season survival due

to a decrease in water lost through respiration. Oxidation of lipids has been shown to be positively correlated with metabolism, suggesting that mosquitoes with low metabolism (aestivating mosquitoes) respire less, and thus lose less water through respiration (Benoit and Denlinger, 2007; Zachariassen et al., 1987). Spiracles are the openings of the tracheal (respiratory) system in insects, and their apertures regulate the trade-off between gas exchange and water loss (Lehmann, 2001). *An. gambiae* has both abdominal and thoracic spiracles, however the abdominal spiracles have better-developed valves, allowing for more effective closing (Vinogradskaya, 1950). The thorax of *An. gambiae* has two sets of spiracles: the anterior, larger mesothoracic spiracles and the posterior, smaller, metathoracic spiracles (Amerasinghe et al., 2002). Size of the spiracle is an important factor in the mosquitoes' ability to adapt to climatic stress (Nagpal et al., 2003). Spiracle aperture is thought to be regulated by a sensory response, as indicated by changes observed in spiracles of live mosquitoes exposed for 5-minutes to high (85% to 95%) and low (< 5%) relative humidity (Krafsur, 1971). In a study of water conservation mechanisms in *Drosophila*, respiratory CO_2 exhalation never fell to zero, suggesting that the spiracles were never closed completely or that at least one spiracle remained open at all times (Gibbs et al., 2003). This finding suggests that spiracle size could, like metabolic rate, be positively correlated with water loss. Indeed the length of thoracic spiracles in xerophilic (dry-loving) species was found to be smaller than in both mesophilic and hygrophilic species (Nagpal et al., 2003). It is not known whether variation in spiracle length can also be cued seasonally *within* a species, via triggers such as changing day length during larval development.

Chemical Markers.

Total Cuticular Hydrocarbon Quantity.

Cuticular hydrocarbons (CHCs) are chemical compounds consisting only of carbon and hydrogen, found on the cuticles of most terrestrial arthropods. CHCs play an important role in the regulation of water loss in insects by affecting the cuticle's permeability to water (Benoit and Denlinger, 2007; Gibbs et al., 1997; Rockey et al., 1991). Diapausing pupae of the flesh fly *Sarcophaga crassipalpis* have been shown to have twice as many hydrocarbons as their non-diapausing counterparts, some of which was deposited on the puparium surface (Yoder et al., 1992). Diapausing pupae of the Hessian fly *Mayetiola destructor* have been shown to have lower rates of transpiration than non-diapausing pupae, and the difference is attributed to increases in cuticular lipids (Benoit et al., 2010). In the grey flesh fly *Sarcophaga bullata*, transpiration rate was found to be higher in non-diapausing pupae than diapausing pupae, and the difference was attributed to the variation in puparium hydrocarbon concentration (Rockey et al., 1991). In *Culex pipiens*, laboratory females reared under winter diapausing conditions had nearly twice the total amount of CHCs as females raised under non-diapausing conditions. When winter diapausing females were exposed to longer day-length (a cue for wetter weather) the amount of hydrocarbons they contained was reduced. Field collections confirmed laboratory findings, with higher CHC content found in females collected in winter than in those collected in the summer (Benoit and Denlinger, 2007). Photoperiodic-induced diapause of *Aedes albopictus* eggs has shown similar results, with diapausing eggs, exposed to a shorter photoperiod, containing one-third more CHCs and

one-half the water loss of non-diapausing eggs (Urbanski et al., 2010).

CHC Relative Abundance.

In addition to increased CHC amount, dry conditions have been associated with extended length of insect CHCs. For example, control populations of female *Drosophila melanogaster* were found to have a relatively high proportion of the shorter 21- and 23-carbon chains while desiccation-selected populations were found to have a relatively higher proportion of the longer 25- and 29-carbon chains (Gibbs et al., 1997). Desiccation-selected *Drosophila melanogaster* were found to reduce water loss by 40% compared to control populations. However excretory water loss explained only 10% of water loss in these *Drosophila*, suggesting that desiccation-resistant flies evolved mechanisms to reduce respiratory and/or CHC water loss (Gibbs et al., 1997) and suggesting that CHC length may be related to cuticle permeability.

Potential Confounders.

Age and insemination status may also affect the CHC profile of insects and therefore could confound the effect of aestivation conditions if their distribution in the compared populations is dissimilar. In a study of *An. gambiae*, no qualitative differences (novel lipids) were found in hydrocarbon profiles of different age-groups, however CHC profiles were found to undergo strong age-related, quantitative changes (changes in relative lipid abundance) (Caputo et al., 2005). Relative abundance of 21-, 23-, 26- and 27-carbon n-alkanes, 3-meC_{25}, 13-, 11-, 9-, 3-meC_{27}, 2-meC_{28} and 3-meC_{29} monomethyl

alkanes have been found to decrease significantly with age in female *An. gambiae* (Caputo et al., 2005). Relative abundance of 21- and 23-carbon n-alkanes was found to decrease with both age and insemination in female *An. gambiae* (Polerstock et al., 2002). Relative abundance of 25-, 26- and 27-carbon n-alkanes was found to decrease with age and increase with insemination in *Aedes aegypti* (Polerstock et al., 2002). Age-related increases in 29-carbon n-alkanes have been reported in *An. farauti* (Hugo *et al.*, 2006). CHC profiles changes that occur as mosquitoes age are thought to play a role in communication of mating status (Polerstock et al., 2002).

Objective.

The primary objective of this research was to identify morphologic and chemical markers capable of distinguishing aestivating and non-aestivating populations of *An. gambiae.* To do this, we reared *An. gambiae* in controlled ambient conditions that simulated dry- or wet-season environments, and then compared the morphologic characteristics and CHC profiles (CHC quantity and type) between the two groups. Morphologic analysis included wing length (a common index of body size) and mesothoracic spiracular index (spiracle length divided by wing length, multiplied by 100). In the CHC profiles, effects of insemination and age were controlled for by analysis of differences between virgin and mated individuals, and between samples collected at different times over the 19-day study.

Hypothesis.

Aestivating mosquitoes are characterized by measurable morphological and chemical traits that serve as adaptations against water loss and can be used as indicators of aestivating and non-aestivating populations.

Predictions.

Morphological Markers.

1. Aestivating females will exhibit longer wing lengths than non-aestivating females.
2. Aestivating females will exhibit smaller mesothoracic spiracular indices than non-aestivating females.

Chemical Markers.

1. Aestivating females will have a larger total amount of CHCs than non-aestivating females.
2. Aestivating females will have, on average, longer CHCs (expressed as longer mean n-alkane retention times) compared with non-aestivating females.
3. Effects of potential confounders: age and insemination status
    a. Relative abundance of n-alkanes will decrease with age for $C \leq 27$ and increase with age for $C \geq 28$
    b. Relative abundance of n-alkanes will decrease with insemination for $C \leq 23$ increase with insemination for $C \geq 25$

c. Relative abundance of monomethyl alkanes will decrease with age

Significance.

Identification of reliable biomarkers for age and insemination status will provide researchers with a classification system to (at least partially) assess *An. gambiae* population structures. Once it is possible to control for the confounding effects of age and insemination status on *An. gambiae* CHC profile, it may be possible to identify reliable biomarkers to distinguish aestivating mosquitoes. Because it is difficult to find *An. gambiae* during the dry season, these biomarkers may be useful to identify and target future aestivators at the end of the wet season. By combining knowledge from CHC profiles and morphological markers with mosquito distribution data, researchers should be able to identify potential aestivation sites (sites containing a higher percentage of aestivating mosquitoes), and focus appropriate vector control strategies when and where they are most needed. Results presented here are pertinent only to nulliparous, non-blood-fed mosquitoes.

CHAPTER II

MATERIALS AND METHODS

Experimental Design.

Rearing Conditions.

This study was conducted in insectaries at the Laboratory of Malaria and Vector Research, NIAID-NIH, using mosquitoes from the NIH-G3 colony. The NIH-G3 colony originated in West Africa and was established in 1972 by the London School of Tropical Medicine. Insectary conditions were maintained at 28°C, with 75% relative humidity, and a 13.5-hour automatically timed photoperiod. HOBO data loggers were used to monitor and record all temperature and relative humidity data. Larvae were raised in plastic pans and were fed daily with commercial fish food. Adult mosquitoes were reared in cylindrical, pint-sized plastic containers placed inside transparent, air and watertight plastic file boxes (Ultimate File Box by Iris, IRIS-UCB-FB, 36.83cm W x 45.47cm L x 27.69cm H), and maintained under controlled environments which mimicked the relative humidity and photoperiod characteristics of mosquito microhabitats during the dry and wet-seasons in Northern Mali. These putative aestivation-inducing and non-aestivation-inducing conditions (referred to in this text as aestivating and non-aestivating conditions) were defined as 77% average relative humidity with 11.5 hours of daylight, and 88% average relative humidity with 13.5 hours of daylight, respectively. Daylight treatments began with newly hatched larvae, and humidity treatment began on the first night after

adult emergence (see Figure 1). Aestivating humidity was controlled using three partially-covered petri dishes (per file box) containing 250g each of drierite™ that were replaced daily. Non-aestivating humidity was controlled using a saturated NaCl solution. (Average aestivating relative humidity was higher than intended due to failure of saturated K_2CO_3 solution to maintain RH at expected level during preliminary trials). For non-aestivators, evening crepuscular period was mimicked by progressive darkening/lightening using an automatic timer (45 minutes in 15-minute intervals). For aestivators, evening crepuscular period was mimicked by progressive darkening/lightening of the containers using cloth covers (two semi-transparent and the third opaque, added at 15 minute intervals). Cloths were removed after insectary darkness was complete, so that all treatments shared the same, automatically timed simulation of morning crepuscular period.

On the day of their emergence, adults were placed into cylindrical pint-sized plastic containers using a manual aspirator. Secured netting covered each pint, such that mosquitoes were contained, yet conditions in each file box were shared by all containers within. Rapid separation prevented unwanted insemination of virgin females, since males are unable to mate during rotation of their genitalia, which occurs during the first 24 hours after emergence. Each file box held four pint-sized containers assigned one of the following groups: 40 males, 40 females, or 20 males and 20 females. Each file box contained a pint of only males, a pint of only females, and two pints with both males and females (see Figure 2). This combination allowed for comparison of morphologic and

chemical markers associated with insemination treatment. While virgin females were separated immediately from mature males and are therefore guaranteed to be virgin, ‘mated’ females were merely exposed to mature males, and are therefore not guaranteed to be mated. Adult mosquitoes were given access to both water and a 10% sugar solution-soaked cotton ball that was refreshed daily.

Sample Collection.

For morphologic analysis, 80 females were randomly collected from each of the two treatment conditions on the day of their emergence. Morphometric analysis was not age dependent, as no change in wing length or spiracle length was expected after emergence. Samples were placed in the freezer overnight. For CHC analysis, 12 females were randomly collected from each of the two daylight regimes on day 1, and four mosquitoes were randomly collected from each pint on days 4, 9, 14, and 19. For mated mosquitoes this sample of four was divided into two males and two females from each pint. Samples were dried in an oven at approximately 55ºC for 24 hours, then placed into plastic screw-cap tubes containing 3-4mL drierite™ covered by cotton. Tubes were sealed with parafilm, and frozen until day of analysis.

Morphologic Analysis.

Wing and mesothoracic spiracle lengths were measured using images analyzed with Lumenara Infinity Software taken on an Olympus CX 41 microscope. Wings were mounted in euparal, and wing length was defined as the distance between the Alular

notch, and point 9 on radius 3 (see Figure 3). After wing removal, mosquitoes were pinned for spiracle length measurement. For pinning, a small amount of clear nail polish was used to fasten the posterior of each mosquito's thorax to the narrow end of a triangular paper point. The pin went through the broad end of the paper point. After attaching the specimen to the point, the pin was inserted into a threaded nut filled with modeling clay, which stabilized pinned mosquitoes under the microscope, and facilitated rotation of the specimen for photography and measurement. Spiracular index is defined as spiracle length divided by wing length, multiplied by 100. The opening of the spiracle is ellipsoid, and spiracle length is measured as the distance across the spiracle's major axis, or transverse diameter (see Figure 4).

CHC Analysis.

Following wing and leg removal (which facilitate CHC extraction), single specimens were submerged in 15µl of heptane for 15 minutes at room temperature. For each specimen, 2µl of sample was injected into a gas chromatograph mass spectrometer (GCMS) without purification. Use of heptane rather than hexane prevented extract evaporation during operation of the auto-sampler. Samples were analyzed at the University of North Carolina at Greensboro (USA) on a Shimadzu GCMS-QP2010S (operating at 0.97kV and acquiring m/z values from 50 to 550). Source and interface temperatures were 200 and 330°C respectively. A 30m RTX-5 column with 0.25-mm diameter, 0.5-µm stationary phase thickness was used with helium as the carrier gas (column head pressure 71.8 kPa, flow rate of 0.73 ml/m, linear velocity as the flow

control mode). Injection temperature was 280°C and injection mode was splitless. After a 1 minute hold, the oven temperature rose from 75 to 160°C at 15°C/min, and then from 160 to 320°C at 5°C/min, with a final hold at 320°C for 20 min. The fifteen largest common peaks between minutes 24 and 34 that were not thought to be contaminants were examined for quantitative variability. Mass spectral libraries used for peak identification included NIST 2005 and WILEY 2007, including supplementary editions. GCMS Post-run Analysis software calculated match percent using an algorithm that compared spectra of the unknown compounds with statistically significant ions from known library spectra.

Statistical Analysis.

Morphologic Markers.

Morphologic data was collected from 160 mosquitoes, 80 reared under each aestivating and non-aestivating conditions. Half of each group of 80 mosquitoes emerged on day 1, the other half on day 2. Dependent variables in morphologic analysis include wing length and spiracular index. Two-way ANOVA was used to analyze the effect of aestivation treatment and emergence (early versus late) on wing length and spiracular index.

Chemical Markers.

Chemical data was collected from 108 mosquitoes, 54 reared under each aestivating and non-aestivating conditions. The majority of the mosquitoes used for chemical analysis were early emergers (emerged on day 1), however 16 of the

mosquitoes sampled (those from containers 5 and 6) were late emergers (emerged on day 2). Dependent variables in CHC analysis include total CHC quantity (CHC-Q), mean n-alkane retention time, and total CHC quantities of individual peaks (denoted by area under the peaks at specific retention times). CHC-Q was calculated for each mosquito sample (using the 15 common peaks) by multiplying peak area by retention time, taking the sum, and then dividing by the mean wing length for that mosquito. Retention time is defined as the time it takes for an injected compound to travel to the detector. Because larger molecules take longer to volatilize, retention time reflects n-alkane chain length and, when combined with quantitative data, can be used as a measure of hydrocarbon relative abundance. Mean n-alkane retention time was calculated for each mosquito (using the 6 n-alkanes identified) by multiplying n-alkane peak area by retention time, taking the sum, and then dividing by the total n-alkane area for that mosquito.

Wing length data was collected for each mosquito used in chemical analysis. Total CHC quantity was calculated for each sample by the adding the total area under 15 specific peaks in each mosquito's CHC profile (see Figure 5). The 15 peaks were the largest common peaks found between retention time (RT) = 24 and 35 minutes, the area of the CHC profile where most peaks were located. The choice to conduct analysis using specific peaks was a result of solvent contamination issues, coupled with the expectation that there would be no qualitative differences in peaks for any samples. Peak identification data was produced from a combination of mass spectrometer library data, literature review, and comparison with retention times from an external hydrocarbon

standard containing pentadecane (C_{15}) and even-numbered alkanes from C_8 to C_{40} (see Figure 5).

To account for mosquito size differences, a CHC index was calculated by dividing the total CHC area for each individual by that individual's average wing length. Mean n-alkane RT was calculated by multiplying each n-alkane RT by the area under its peak, calculating the sum of those numbers, and then dividing by the total area for all n-alkanes. Each set of two data collections from the same container, pint (insemination status), and age (collected on the same day) were averaged, and counted as a single data point. Split-split-plot ANOVA was used to analyze the main effect and statistical interactions between aestivation treatment, age, and insemination status on total CHC quantity, mean n-alkane retention time, and the total quantity of each individual peak. For each ANOVA, the magnitude of the differences was estimated with 95% confidence and a lower bound of the interval greater than 0 provided statistical evidence to support the predictions (ie: the rejection region for the null hypothesis was defined as $p \leq 0.05$). For analysis of total CHC quantity and mean n-alkane retention time, insignificant factors were removed from the models and the models were rerun. In order to maintain consistency for comparisons between individual peaks, all factors, including insignificant ones, were kept in the models for analysis of individual peak CHC quantity. Additional 2-way ANOVA were used to analyze the effect of aestivating conditions and age on CHC quantity for virgin and mated individuals independently. The p-values for these ANOVA are displayed with R^2 values in figures 8, 11, and 12.

CHAPTER III

RESULTS

Morphologic Markers.

Wing Length.

Mean wing length of newly emerged females reared under aestivating conditions (decreased photoperiod) was significantly larger than that of females reared under non-aestivating conditions (Figure 6, Table 1). After accounting for emergence day, the mean wing length of aestivators is estimated to be 5.8% greater than non-aestivators. We also found significant evidence of an emergence day effect, with early emerging mosquitoes being significantly larger (2.9%) than late emergers (Figure 6, Table 1). We fit the model and performed a test for interaction. The effect of photoperiod on wing length is greater in early emergers than in late emergers (Figure 6, Table 1).

Spiracle Length.

Mean spiracular index (SI) of newly emerged females reared under aestivating conditions (decreased photoperiod) was significantly larger than that of females reared under non-aestivating conditions (Figure 7, Table 2). The mean SI of aestivators is estimated to be 5.5% greater than in non-aestivators. We also found significant evidence of an emergence day effect, with early emerging mosquitoes having a significantly larger SI (7.3%) than late emergers (Figure 7, Table 2). The effect of photoperiod on SI was not

different between early and late emergers (non-significant treatment-by-emergence day interaction p=0.92, data not shown).

Chemical Markers.

Total CHC Quantity.

Mean total CHC quantity standardized by wing length (CHC-Q) of females reared under aestivating conditions was significantly larger than that of females raised under non-aestivating conditions (Figure 8, Table 3). The mean total CHC-Q of the aestivators is estimated to be 28% greater than in non-aestivators (Table 4). There is suggestive but inconclusive evidence that mean total CHC-Q of inseminated females was larger than that of virgin females (Figure 8, Table 3). Though results were not significant due to high error, mean total CHC-Q of inseminated females is estimated to be 58% greater than in virgins (Table 4). Mean total CHC-Q increased significantly with age (Figure 8, Table 3). The mean total CHC-Q of 19-day-old females is estimated to be 152% greater than in 1-day-old females (Table 4). Results from 2-way ANOVA on the effect of aestivating conditions and age on total CHC quantity reveal that the effect of treatment is significant for virgins and insignificant for mated individuals (Figure 8).

N-alkane Relative Abundance.

Mean n-alkane retention time (RT) increased significantly with age, and was significantly larger in non-aestivators than aestivators (Figure 9, Table 5). A strong treatment-by-age interaction was also observed, with mean RT of non-aestivators

increasing at a faster rate than that of aestivators (Figure 9, Table 5). Insemination status did not significantly affect mean n-alkane RT ($p = 0.43$).

Individual Peak Relative Abundance.

The areas of all 15 CHC peaks were greater in mated mosquitoes and in mosquitoes exposed to aestivating conditions (Table 6). Effects of mating were significant in peaks 2, 3, 5, 9, 10 and 14 (Table 6). Effects of aestivating conditions were significant in peaks 3, 5, and 11 (Table 6). CHC quantity (as measured by peak area) also tended to increase with age, as observed in peaks 2, 4, 6, 7, 9, 11, 13, 14, and 15. This age effect was significant in peaks 6, 9, 13 and 15, and was not observed in any of the branched hydrocarbons (peaks 8, 10 and 12) (Figure 10, Table 6). In peaks 3 and 5, there were significant treatment-by-age interactions, where CHC quantity increased significantly in aestivators and decreased significantly in non-aestivators over time (Figures 11 and 12, Table 6). Peak 9 was confounded by more than one significant effect. In peak 9, the rate of CHC quantity increase was significantly lower in virgin, non-aestivating individuals (Table 6). Peak 10 was the only identified peak that increased significantly with insemination, and was not confounded by other variables (Figure 13, Table 6). Results from 2-way ANOVA on the effect of aestivating conditions and age on total CHC quantity reveal that the effect of treatment is significant for virgins and insignificant for mated individuals (Figures 11 and 12).

Peak Identification.

Peaks 3, 5, 6, 8, 9, 10, 12, 13, and 14 were identified based on fragmentation spectra matches in the NIST and WILEY libraries (Table 7). The most likely structural assignments from among the possible matches were chosen based on a literature review of mosquito cuticular hydrocarbons (Benoit and Denlinger, 2007; Caputo et al., 2005; Caputo et al., 2007; Hugo et al., 2006; Polerstock et al., 2002; Rockey et al., 1991), and a comparison of retention times with an external hydrocarbon standard. Tentative peak identifications include 6 n-alkanes, 2 monomethyl alkanes, and 1 dimethyl alkane.

CHAPTER IV

DISCUSSION

Morphologic Markers.

Body Size.

As predicted, mean body size (indexed by wing length) was larger in mosquitoes exposed to aestivating conditions. This finding is consistent with findings from several previous studies linking large body size to aestivating conditions (Benoit and Denlinger, 2007; Kikukawa et al., 1984; Lanciani, 1992; Lyimo et al., 1992) and linking increased growth rate to time stress (Abrams et al., 1996), as may be experienced by larvae when water sources begin to dry out at the end of the wet season. The 5.8% increase in mean wing length found here is consistent with previous research, which found wing length to be greater in females exposed to artificial short photoperiods (8L:16D compared to 16L:8D) by 5.1% (June collection) and 9.2% (January collection, time of natural diapause) (Lanciani, 1992). Size of adults at emergence has been associated with environmental factors including temperature, photoperiod, food availability, and larval density (Corkum and Hanes, 1992; Hanes and Ciborowski, 1992).

Increase in body size of mosquitoes exposed to aestivating conditions is expected to have evolved as an adaptation for the suppression of transcuticular water loss in larger individuals through a decrease in the body's surface area to volume ratio (Benoit and

Denlinger, 2007; Day and Lawrence, 2000). Any increased energetic cost of flying with a larger body is likely mitigated by a reduction in flight activity expected during aestivation and diapause, when metabolic rate is decreased (Benoit and Denlinger, 2007). Compared to other animals, flying insects have very high mass-specific metabolic rates (metabolic rate per unit tissue) (Suarez, 2000) meaning that any increase in mass leads to a substantial increase in metabolism during flight. Since metabolism must be depressed during aestivation to suppress spiracular water loss, mosquito body size must remain in a delicate balance between being large enough to decrease surface area to volume ratio, and being small enough to minimize metabolic rate during flight.

Emergence day was also found to have a significant effect on mosquito body size, with early emergers being significantly larger than late emergers. This effect was measured over only two days of emergence, and may be even more significant when measured over all emergence days. While this effect has been documented in other arthropods (Corkum et al., 1997; Sweeney and Vannote, 1981), this study provides, to our knowledge, the first evidence of this effect in *An. gambiae*. Seasonal and stress-induced changes in juvenile growth rate are well documented and allow for such adaptations as early maturation or increased body size (Abrams et al., 1996). Early emergence is especially advantageous to aestivators, who are likely to emerge at the beginning of the dry season when surface water needed for larval development is drying up, and when the threat of pre-emergence desiccation is most severe. In this study, the effect of emergence day on adult size was significantly more pronounced in aestivators

than in non-aestivators. Because larval density and access to food resources should not have differed significantly between treatment conditions in this experiment, the interaction observed can be attributed to the difference in photoperiod.

Spiracular Index.

Contrary to predictions, spiracular index was larger in mosquitoes exposed to aestivating conditions than those exposed to non-aestivating conditions. This significant increase in mean SI of aestivators (5.5%) may be a result of reduced metabolism and consequent reduction in spiracular water loss, variation in spiracle shape (ie: the spiracle is longer but not larger overall), occupation by mosquitoes of humid microhabitats, or need for significantly increased gas exchange during flight with a larger body. It is also possible that wing length is an inadequate indication of surface area, which is the specific aspect of body size most relevant to analysis of spiracle length.

Threat of desiccation in insects is most severe during flight, when spiracles must remain open to allow increased O_2 and CO_2 flux needed to maintain higher metabolic activity (Lehmann, 2001). The rate of metabolism of insects during flight increases up to 50-fold compared to that at rest (Roff and Fairbairn, 1991). Since water is lost rapidly through open spiracles, mosquitoes facing desiccation stress conserve water through reduction of flight activity (Lehmann, 2001), explaining in part the apparent decrease in the *An. gambiae* population during the dry season in the Sahel region of Mali (Lehmann et al., 2010). Since energy is required to activate the spiracular closer muscles (Lighton,

1996), less active, lower metabolizing aestivating mosquitoes must either have evolved to develop differences in spiracle size and shape (during larval development), or exert more energy than non-aestivators to hold spiracles closed (as adults). Longer SIs in mosquitoes exposed to aestivating conditions may suggest that sensory control of spiracle aperture is more important than spiracle length in reduction of spiracular gas exchange and water loss. Since energy is required to activate the spiracular closer muscles, increased need for sensory control of spiracle aperture in aestivating mosquitoes might select for narrower spiracles, which would reduce energy expenditure. Narrow spiracles may be longer to allow for maintenance of sufficient gas exchange with reduced energy input.

It is also possible that *An. gambiae* are able to minimize water loss during the dry season by selecting microhabitats within microclimates characterized by high relative humidity. If moderately high relative humidity microhabitats are available to and used by *An. gambiae,* then reduction in spiracle size may not be as important an adaptation for survival during the dry season, especially if they are able to reduce their respiratory water loss by slowing their metabolism. However this still does not explain the increase in spiracular index observed in aestivating mosquitoes. One possibility is that aestivators must fly farther before and/or after aestivation (possibly in search of a humid microhabitat), and thus require larger spiracles to support the significantly increased gas exchange required during flight with a larger body.

Because spiracle length quantifies a dimension of the two-dimensional spiracular opening on the surface of mosquito, surface area is the specific aspect of body size most accurate for standardization of spiracle length. Studies have shown a high positive correlation between wing length and body mass (Lounibos et al., 1995), however body mass is more closely related to volume than surface area. Should wing length be more closely associated with volume than surface area, we would expect to see increased wing length resulting in smaller than expected SIs for larger individuals, and thus, for aestivators. This is due to the nature of the relationship between volume and surface area: as an object gets larger, the volume increases more rapidly than the surface area. If wing length is a better indicator of volume than surface area, the difference in SI found in this study may in fact be underestimated, as spiracle length of larger mosquitoes, such as those exposed to aestivating conditions, was divided by a disproportionately larger wing length.

Chemical Markers.

Total CHC Quantity.

As predicted, total CHC-Q was higher in mosquitoes exposed to aestivating conditions. This is likely due to CHCs' role in regulation of water loss in insects, and the negative correlation between CHC quantity and cuticular permeability (Benoit and Denlinger, 2007; Gibbs et al., 1997), which is particularly important to insects facing desiccation stress. The results presented here are consistent with findings from previous studies, which indicate a total CHC quantity increase of approximately 30-50% for

mosquitoes exposed to aestivation conditions. (Benoit and Denlinger, 2007; Urbanski et al., 2010).

Total CHC-Q also increased in mated individuals and with age. While mating was observed between live specimens, experimental set-up did not guarantee that all females in mixed-sex pints were mated. Maximum mating activity of related species *An. culicifacies* has been found to take place between age 5-12 days for females and age 5-7 days for males (Mahmood and Reisen, 1994). Optimum insemination age for *An. gambiae* and *An. arabiensis* has been found to be 7 days for both males and females (Verhoek and Takken, 1994). In addition, insemination rate of *An. gambiae* in the laboratory has been found to be consistently low (72% maximum) relative to other species such as *An. arabiensis* (96%) (Verhoek and Takken, 1994). The possibility that 'mated' individuals may actually still be virgin at the time of sample collection likely contributes to the greater variability observed in CHC profiles of 'mated' individuals than in those of virgins (see R^2 values, 95% confidence intervals, and ranges displayed in Figures 8, 11, 12, and 13). This possibility was further analyzed using 2-way ANOVA for the effect of aestivating conditions and age on CHC quantity for virgin and mated individuals independently, and is demonstrated in the significance and non-significance of the effect of aestivation conditions on CHC quantity in virgins and mated individuals respectively. High variability in 'mated' samples is likely an artifact of experimental setup (p-values for the 2-way ANOVA are displayed in Figures 8, 11, and 12). The effect

of insemination and age on CHC-Q is discussed further below, with respect to changes in quantity of individual peaks.

N-alkane Relative Abundance.

Mean n-alkane RT was significantly lower in mosquitoes exposed to aestivating conditions. This is not consistent with our predictions, which were based on findings of increased CHC length in desiccation-selected *Drosophila* (Gibbs et al., 1997). It is possible that production of shorter CHCs allows for an increase in total CHC quantity, and that total CHC quantity is more important than hydrocarbon length for suppression of transcuticular water loss. It is also possible that smaller n-alkanes compact better, and are therefore more effective at sealing the cuticle. Mean n-alkane RT did increase with age as predicted, which is consistent with findings from *An. gambiae*, *Aedes aegypti* (Polerstock et al., 2002), and *An. farauti* (Hugo et al., 2006).

Individual Peak Relative Abundance.

Mean CHC-Q pertaining to peak 11 (ie: area under peak 11 divided by the mean wing length) was found to be significantly higher in aestivating mosquitoes than in non-aestivating mosquitoes, and was not found to vary significantly with age or insemination. However identity of peak 11 was not determined. Mean CHC-Q pertaining to peaks 3 and 5 (identified as pentacosane and heptacosane) decreased significantly with age in non-aestivators, and increased significantly with age in aestivators. Peaks 3 and 5 also increased significantly with insemination. Once age and insemination status are

determined, peaks 3 and 5 can be used to infer the aestivation status of nulliparous, non-blood fed female *An. gambiae*.

Mean CHC-Q of 3 of the 6 n-alkanes (peaks 6, 9, and 13) and of peak 15 increased significantly with age. Because they were identified and because their CHC-Qs did not vary significantly with aestivation treatment or insemination, peaks 6 and 13 make the most reliable biomarkers for determination of age in populations of nulliparous, non-blood fed females. Mean CHC-Q of peaks 2, 3, 5, 9, 10 and 14 were significantly greater in mated individuals than in virgins. However identity of peak 2 was not determined, peaks 3, 5 and 9 also varied significantly with age, and there is inconclusive evidence suggesting that peak 14 may be higher in aestivators than in non-aestivators. Peak 10 therefore makes the most reliable biomarker for distinguishing between mated and virgin populations of nulliparous females.

Once age and insemination status of a population are determined (using peaks 6, & 13, and 10 respectively), aestivation status can be assessed using peaks 3 and 5. For example, as indicated in Figure 12, if a female is determined to be a 15 day-old virgin, a peak 5 CHC-Q of around 20,000 would classify her as a non-aestivator, while and peak 5 CHC-Q of around 60,000 would classify her as an aestivator. Analysis of relative abundance of individual peaks may serve as a reliable method for determining population structures, including aestivation status, insemination status, and age of nulliparous female *An. gambiae.*

In peaks 3 and 5, results from the non-aestivators support our prediction that relative abundance of n-alkanes ($C \leq 27$) decrease with age, and these results are consistent with previous findings (Caputo et al., 2005; Polerstock et al., 2002). A comparison of rearing methodology reveals that Polerstock rearing conditions (RH: 80%, photoperiod: 14h, crepuscular period: n/a) more closely resemble those of this study's non-aestivating group (RH: 88%, photoperiod: 13.5h, crepuscular period: 0.75h), while Caputo rearing conditions (RH: 75 ± 10%, photoperiod: 12h, crepuscular period: 1h) more closely resemble those of this study's aestivating group (RH: 77%, photoperiod: 11.5h, crepuscular period: 0.5h) (Caputo et al., 2005; Polerstock et al., 2002). Temperature was consistent (27 ± 1°C) in all three studies. The unexpected increase of CHC-Q with age in aestivators for peaks 3 and 5 may be explained by the greater photoperiod in both the Polerstock and Caputo studies compared to the aestivating group here, especially when the extended crepuscular periods are considered. The increase of peak 9 CHC-Q with age is consistent with previous findings (Hugo et al., 2006). No monomethyl alkanes were identified in this study, so we are unable to offer analysis regarding the effect of age on monomethyl relative abundance. The increase in CHC-Q of peaks 3, 5, 9, 10, and 14 in mated individuals supports our prediction, and is consistent with previous findings (Polerstock et al., 2002).

Mass Spectrometry Data.

It is important to note that all structural assignments based on GCMS chromatograms are tentative. Because mass spectrometry peak identification is

based on fragmentation alone, peak identification presented here was enhanced with both literature review and a comparison of retention times to an external hydrocarbon standard. Compounds with similar structures can have very similar fragmentation patterns, and therefore relying on fragmentation patterns alone may lead to misidentification of compounds. Improving peak-identification methodology with literature review and a comparison of retention times enhanced our ability to accurately identify peaks. Unknown peaks may be contaminants, or may just not be in the mass spectrometry databases used for this study.

Summary and Way Forward.

The research presented in this study may enhance disease ecologists' understanding of morphologic and chemical biomarkers capable of distinguishing nulliparous, non-blood-fed aestivating mosquitoes. Increased understanding of the dry season survival mechanisms of *An. gambiae* in semi-arid regions could benefit vector control efforts by identifying weak links in the transmission cycle of malaria. This study also provides data useful in the assessment of malaria-vector population structures, including biomarkers for age and insemination status. By combining knowledge from morphological markers and CHC profiles with mosquito distribution data, researchers should be able to identify aestivation sites (sites containing a higher percentage of aestivating mosquitoes), and focus appropriate vector control strategies where they are most needed.

Research regarding the effects of aestivation conditions on width and total area of the spiracular opening may be useful in confirming increased spiracular index in aestivating mosquitoes, and in improving our understanding of the ultimate causes of this phenomenon. One significant advantage of morphologic biomarkers over chemical biomarkers is the relative ease with which they can be measured. While chemical biomarkers are widely used and respected, they can be costly in terms of both time input and equipment required. In contrast, morphologic biomarkers can be analyzed quickly, using less expensive equipment. In addition, while chemical analysis is limited by small sample volume and is somewhat destructive to the sample, morphologic analysis is less invasive, and can be easily repeated and confirmed for the same samples. Nevertheless, chemical markers reveal insights about dry-season survival strategies that morphologic markers cannot, and both are useful in the understanding of malaria vector survival.

It has been hypothesized that aestivating female *An. gambiae* may favor sugar meals over blood meals during periods of dormancy as a result of the increased metabolism required for blood digestion (Huestis et al., In Press). However, for full applicability to field-collected specimens, further research is needed to understand the effects of aestivation conditions and age on blood-fed, parous mosquitoes. A future study modeled after this one, that includes analysis of the effect of blood-feeding status on morphologic and chemical biomarkers, could serve to both substantiate these results and enhance our ability to calibrate population structures of mosquitoes as we are likely to encounter them in the field. Once the effects of aestivation conditions and age on

morphologic and chemical biomarkers of parous, blood-fed mosquitoes are more clearly understood, analysis of biomarkers in field-collected specimens will be warranted.

With malaria claiming the lives of nearly a million people every year, it is clear that we cannot afford to underestimate the significance of our ecological understanding of the disease's primary vector, *An. gambiae*. Preventative strategies are especially important since most victims of malaria are impoverished, lacking adequate access to both medicine and health care. While large-scale prevention of malaria contraction is, at the very least, a lofty aspiration, advances in scientific understanding of population structures and development of improved vector control strategies do have the potential to make a profound impact on prevalence of the disease. This study offers a small but significant development in the fight against malaria, one of the oldest, most notorious diseases to ever threaten the well-being of our species.

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APPENDIX A: TABLES

Table 1. ANOVA for Effect of Increased Photoperiod and Late Emergence on *An. gambiae* Body Size

Model	**Unstandardized Coefficients**				**95.0% Confidence Interval for B**	
	B	Std. Error	t	Sig.	Lower Bound	Upper Bound
(Constant)	28.78	0.14	210.20	0.000	28.51	29.05
Photoperiod	-1.56	0.20	-8.00	0.000	-1.94	-1.17
Emergence Day	-1.15	0.19	-5.92	0.000	-1.53	-0.76
Interaction	0.75	0.27	2.75	0.007	0.21	1.29

Table 2. ANOVA for Effect of Increased Photoperiod and Late Emergence on *An. gambiae* Spiracular Index

Model	**Unstandardized Coefficients**				**95.0% Confidence Interval for B**	
	B	Std. Error	t	Sig.	Lower Bound	Upper Bound
(Constant)	3.26	0.03	110.39	0.000	3.21	3.32
Photoperiod	-0.16	0.03	-4.73	0.000	-0.22	-0.09
Emergence Day	-0.22	0.03	-6.31	0.000	-0.28	-0.15

Table 3. Split-split plot ANOVA for Effect of Aestivation Conditions, Insemination, and Age on Total CHC quantity (standardized by wing length)

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F	p
Aestivation	2.99E+11	1	2.99E+11	6.30	0.05
Insemination	4.78E+11	1	4.78E+11	5.52	0.07
Age	2.58E+11	3	8.60E+10	4.24	0.01

Table 4. Summary of Mean Total CHC Quantities (standardized by wing length)

Mosquito Description	Mean Total CHC-Q	Difference	Percent Increase
Non Aestivating	4.93E+5		
Aestivating	6.33E+5	1.40E+5	28
Virgin	4.53E+5		
Mated	7.14E+5	2.61E+5	58
Day 1	2.92E+5		
Day 4	5.07E+5		
Day 9	6.15E+5		
Day 14	6.10E+5		
Day 19	7.34E+5	4.42E+5*	152*

* From Day 1 to Day 19

Table 5. Split-split plot ANOVA for Effect of Aestivation Conditions, Insemination, Age, and Interactions on n-alkane Relative Abundance

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F	p
Aestivation	0.91	1	0.91	9.01	0.04
Age	1.54	3	0.51	4.62	0.007
Aestivation *Age	1.63	3	0.54	4.91	0.005

Table 6. Mean Individual Peak CHC Quantity with Respect to Aestivation Conditions, Insemination, and Age, Including Significance from Individual Peak split-split plot ANOVA

Peak	Aestivating	Non-aestivating	Mated	Virgin	1 Day	4 Days	9 Days	14 Days	19 Days
1	2.5 E+4	2.2E+4	2.5E+4	2.3E+4	2.2E+4	2.4E+4	2.5E+4	2.3E+4	2.3E+4
2	9.8 E+3	9.4E+3	1.1E+4*	8.5E+3	6.1E+3	9.8E+3	1.0E+4	1.1E+4	1.0E+4
	2.1 E+4*	1.1E+4	2.0E+4*	1.1E+4	8.5E+3	2.1E+4	1.4E+4	1.4E+4	1.7E+4
	Virgin Non-aestivating				2.6E+3	1.6E+4	3.1E+3	2.0E+3	2.4E+3
	Mated Non-aestivating				-	3.4E+4	1.5E+4	7.2E+3	1.3E+4
	Virgin Aestivating				1.2E+4	1.4E+4	2.1E+4	1.7E+4	1.9E+4
3†	Mated Aestivating				-	2.2E+4	1.7E+4	2.8E+4	2.7E+4
4	9.6E+3	8.8E+3	1.2E+4	7.4E+3	4.5E+3	9.7E+3	9.3E+3	9.7E+3	1.2E+4
	6.0 E+4*	3.9E+4	6.2E+4*	4.1E+4	3.4E+4	5.3E+4	5.2E+4	4.6E+4	6.0E+4
	Virgin Non-aestivating				2.7E+4	3.7E+4	2.2E+4	2.2E+4	1.6E+4
	Mated Non-aestivating				-	6.9E+4	5.2E+4	3.7E+4	5.7E+4
	Virgin Aestivating				3.7E+4	4.3E+4	6.9E+4	5.8E+4	6.9E+4
5†	Mated Aestivating				-	6.5E+4	6.3E+4	6.9E+4	8.3E+4
6	4.2E+3	3.2E+3	5.4E+3	2.4E+3	1.3E+3*	2.7E+3	3.8E+3	3.8E+3	6.3E+3
7	1.8E+3°	1.6E+4	2.0E+4	1.5E+4	1.2E+4	1.7E+4	1.7E+4	1.8E+4	2.0E+4
8	7.9E+3	5.0E+3	9.3E+3°	4.4E+3	4.2E+3	8.9E+3	6.6E+3	5.4E+3	6.9E+3
9	8.2E+3°	6.2E+4	9.5E+4*	5.5E+4	1.3E+4*	4.1E+4	6.7E+4	9.5E+4	1.3E+5
10	9.9E+3	7.8E+3	1.2E+4*	6.4E+3	4.8E+3	1.0E+4	9.6E+3	7.4E+3	1.1E+4
11	2.0E+4*	1.7E+4	2.2E+4	1.6E+4	1.1E+4	1.8E+4	2.0E+4	1.9E+4	2.1E+4
12	1.3E+4°	1.0E+4	1.6E+4	7.9E+3	6.5E+3	1.6E+4	1.2E+4	9.7E+3	1.2E+4
13	5.4E+4	4.6E+4	6.7E+4	3.8E+4	1.6E+4*	3.7E+4	5.3E+4	5.9E+4	7.7E+4
14	8.0E+4°	6.2E+4	9.5E+4*	5.2E+4	2.5E+4	7.4E+4	8.1E+4	7.3E+4	8.9E+4
15	2.2E+5	1.7E+5	2.4E+5	1.7E+5	1.2E+5*	1.6E+5	2.2E+5	2.2E+5	2.4E+5

***Astrix indicates a significant evidence of an effect ($p < 0.05$) and °degree symbol indicates suggestive evidence of an effect ($0.05 < p < 0.09$) for the factor (Aestivation, Insemination, or Age) indicated in that column. A †cross in the left-hand column indicates a significant ($p < 0.05$) treatment-by-age interaction.**

Table 7. Individual Peak Identification with Approximate Retention Time (in minutes) and Percent Library Match

Peak #	Approximate RT	Identification	Formula	Library Match
1	23.9	-	-	-
2	24.0	-	-	-
3	25.3	Pentacosane	$C_{25}H_{52}$	94%
4	26.2	-	-	-
5	28.3	Heptacosane	$C_{27}H_{56}$	94%
6	29.7	Octacosane	$C_{28}H_{58}$	88%
7	30.1	-	-	-
8	30.7	1-Hexacosene	$C_{26}H_{52}$	84%
9	31.0	Nonacosane	$C_{29}H_{60}$	96%
10	31.4	2,3-dimethylnonadecane	$C_{21}H_{44}$	81%
11	31.9	-	-	-
12	33.4	9-Hexacosene	$C_{26}H_{52}$	94%
13	33.6	Hentriacontane	$C_{31}H_{64}$	79%
14	34.0	Dotriacontane	$C_{32}H_{66}$	92%
15	34.3	-	-	-

APPENDIX B: FIGURES

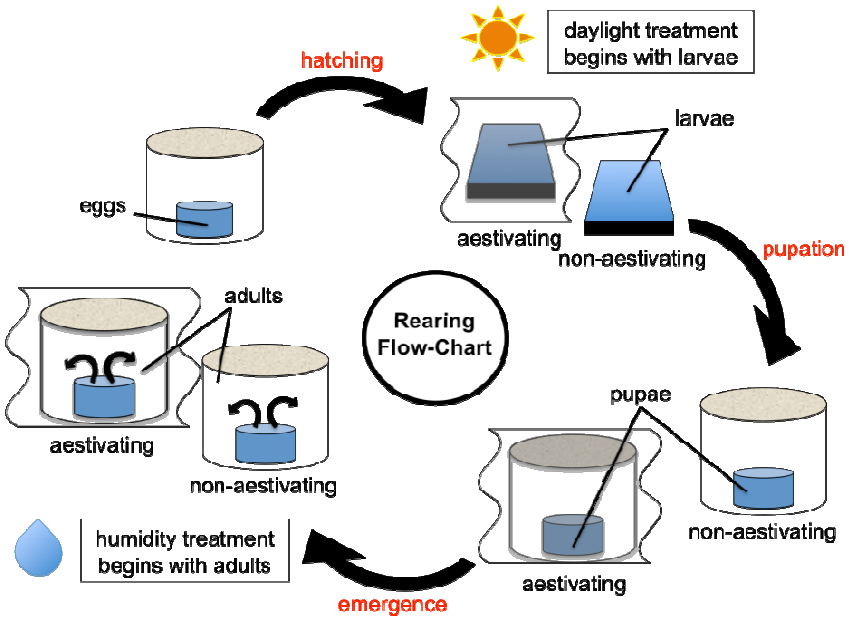

Figure 1. Rearing flow chart illustrating developmental stages of *An. gambiae* and time of initiation for daylight and humidity treatments

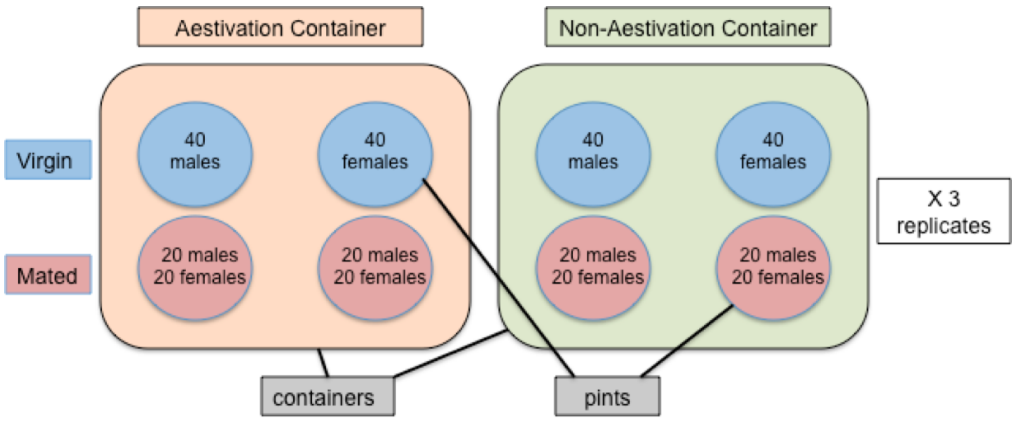

Figure 2. Experimental setup for adult mosquitoes, where large rectangles represent filebox containers and circles represent pints.

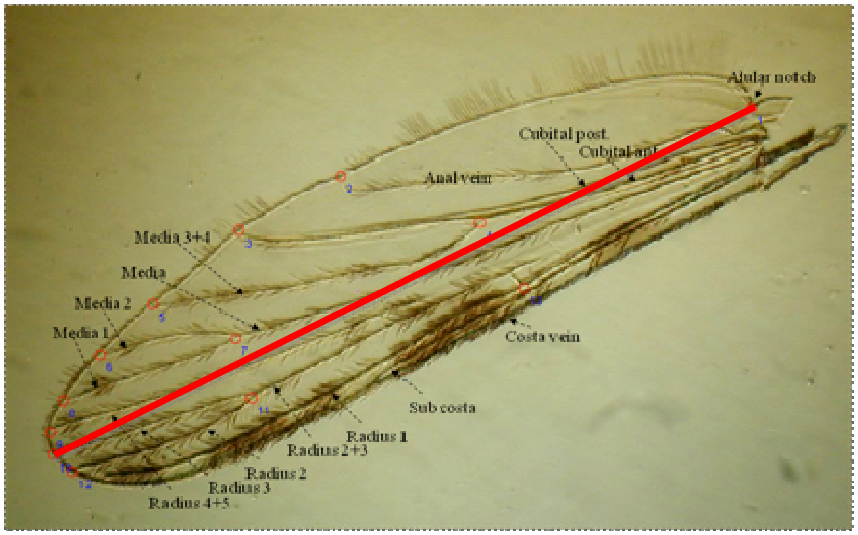

Figure 3. Wing Landmarks (Photo by Lehmann Lab, Laboratory of Malaria and Vector Research, NIAID, NIH).

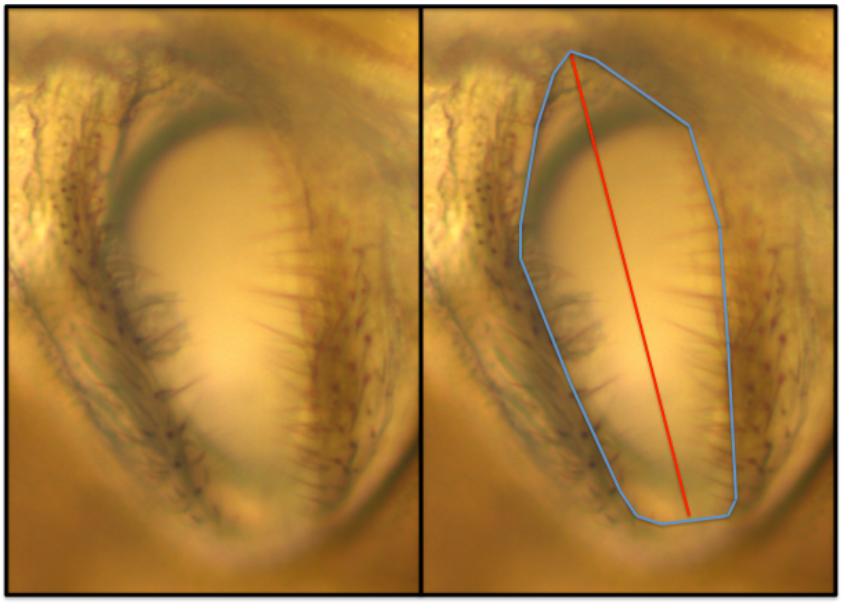

Figure 4. *An. gambiae* spiracle; Blue line indicates boundaries of spiracular opening, red line indicates transverse diameter (spiracle length)

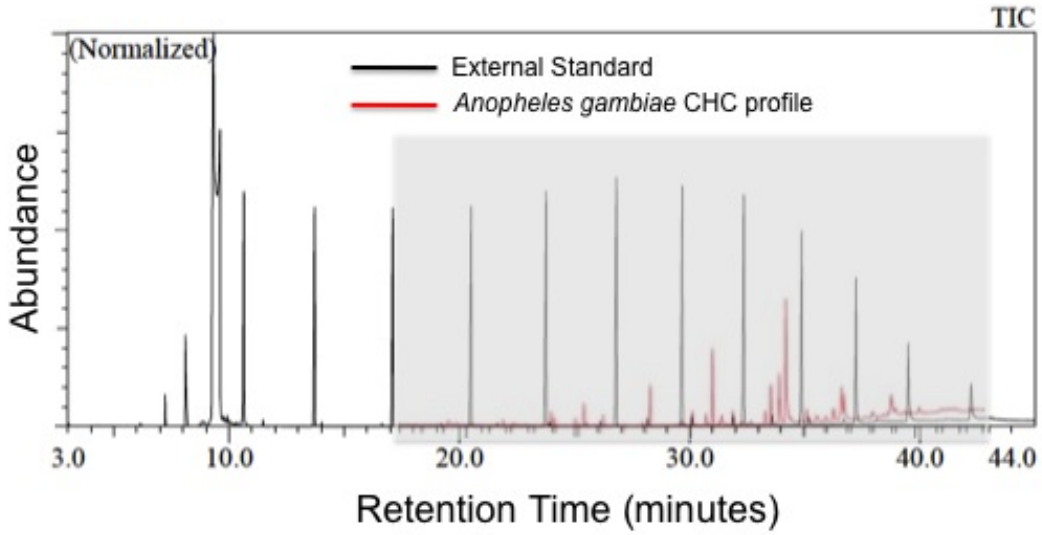

Figure 5. Overlay of external standard (run separately from mosquito samples) and *An. gambiae* GCMS chromatograms.

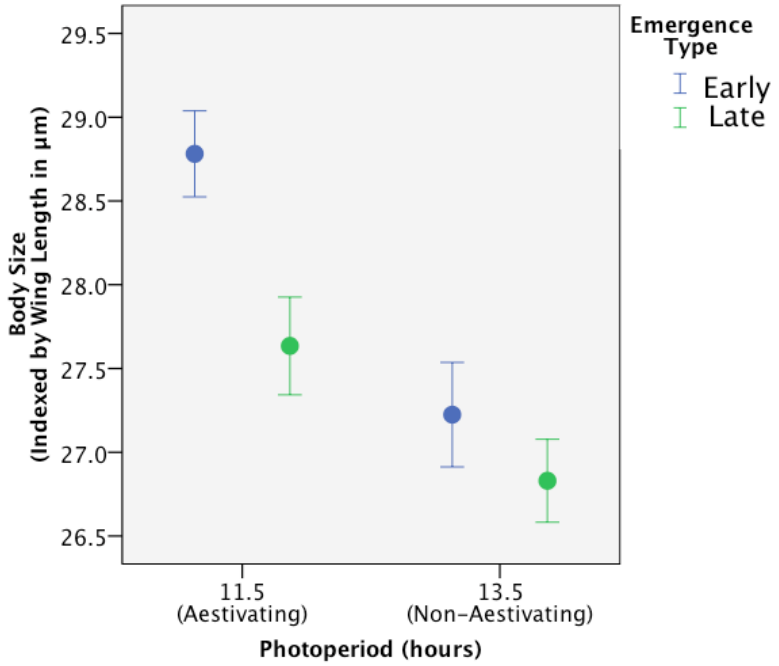

Figure 6. Effect of Aestivation Conditions (Increased Photoperiod) and Emergence on *An. gambiae* Body Size, with 95% Confidence Intervals

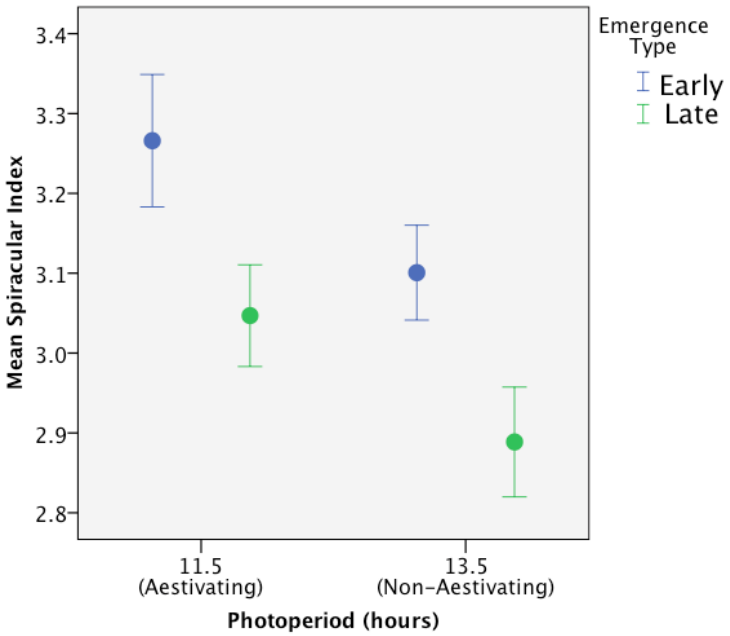

Figure 7. Effect of Aestivation Conditions (Increased Photoperiod) and Emergence on *An. gambiae* Spiracular Index, with 95% Confidence Intervals

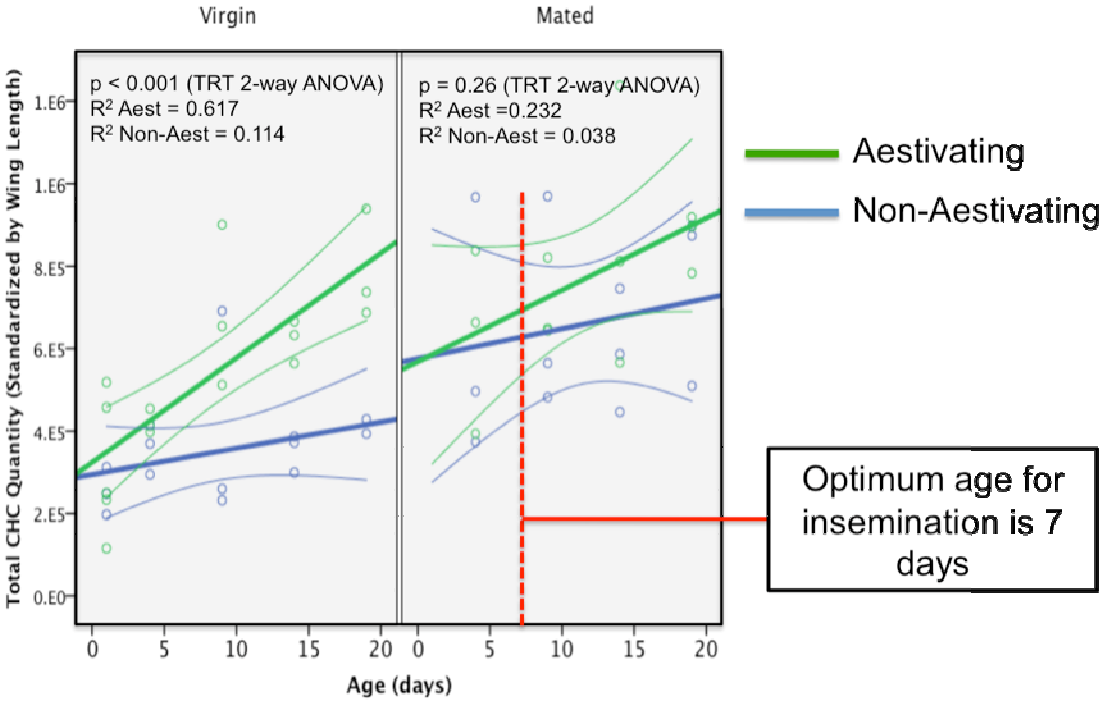

Figure 8. Effect of Aestivation Conditions, Insemination, and Age on *An. gambiae* Total CHC Quantity (standardized for size) with 95% Confidence Intervals; Aestivation Conditions defined as Short Photoperiod

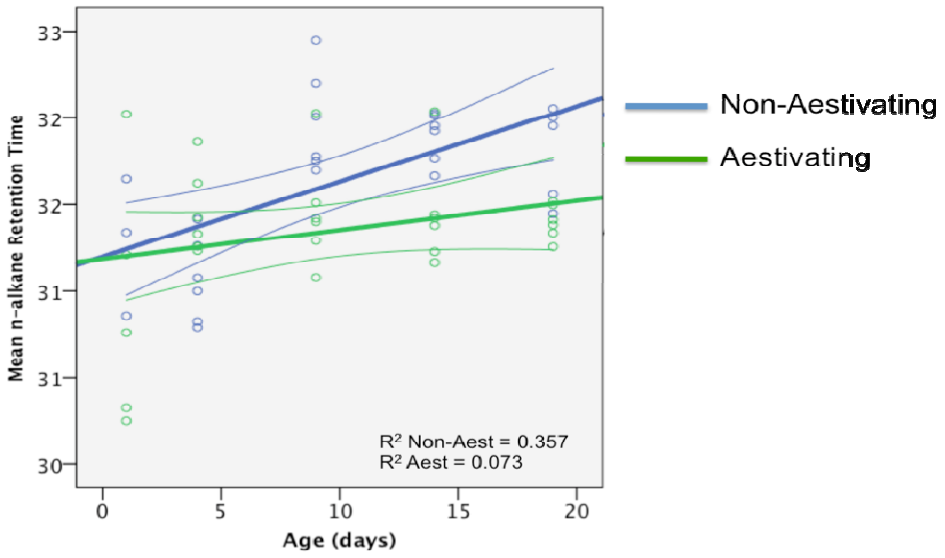

Figure 9. Effect of Aestivation Conditions and Age on *An. gambiae* Mean n-alkane Retention Time, with 95% Confidence Intervals; Aestivation Conditions defined as Short Photoperiod

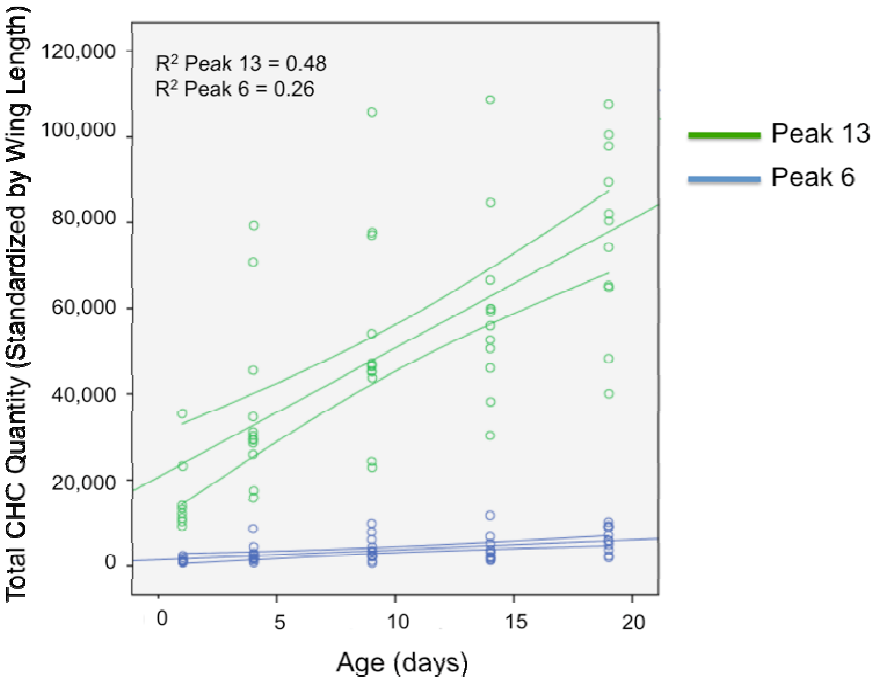

Figure 10. Effect of Age on Mean *An. gambiae* Peak 6 and 13 CHC Quantity, with 95% Confidence Intervals

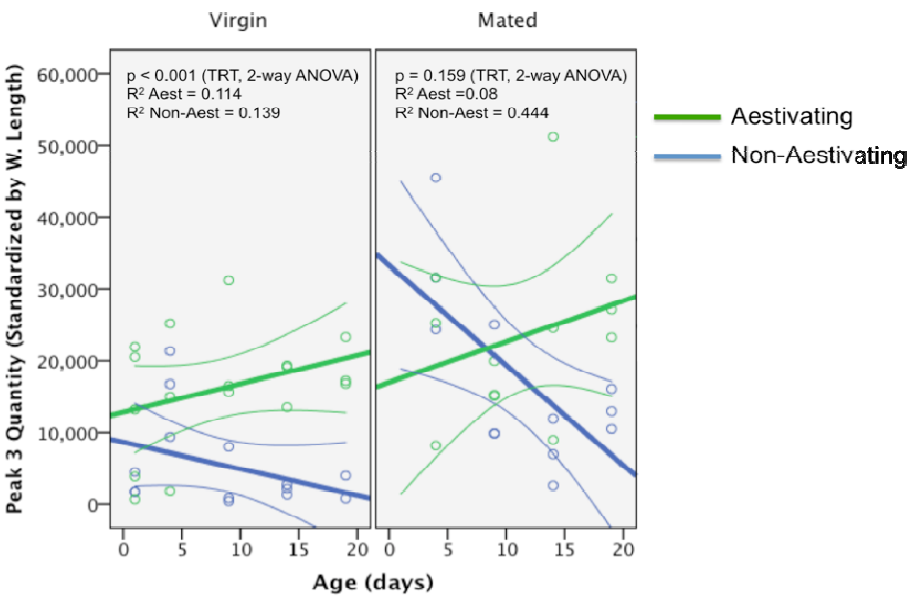

Figure 11. Effect of Aestivation Conditions, Insemination, and Age on *An. gambiae* Peak 3 Quantity with 95% Confidence Intervals; Aestivation Conditions defined as Short Photoperiod

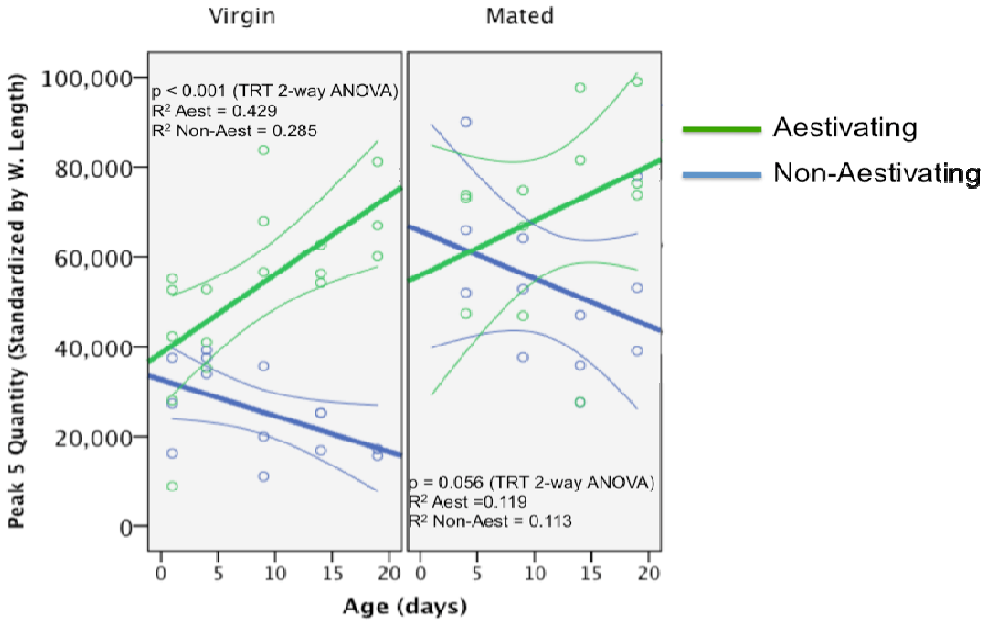

Figure 12. Effect of Aestivation Conditions, Insemination, and Age on *An. gambiae* Peak 5 Quantity with 95% Confidence Intervals; Aestivation Conditions defined as Short Photoperiod

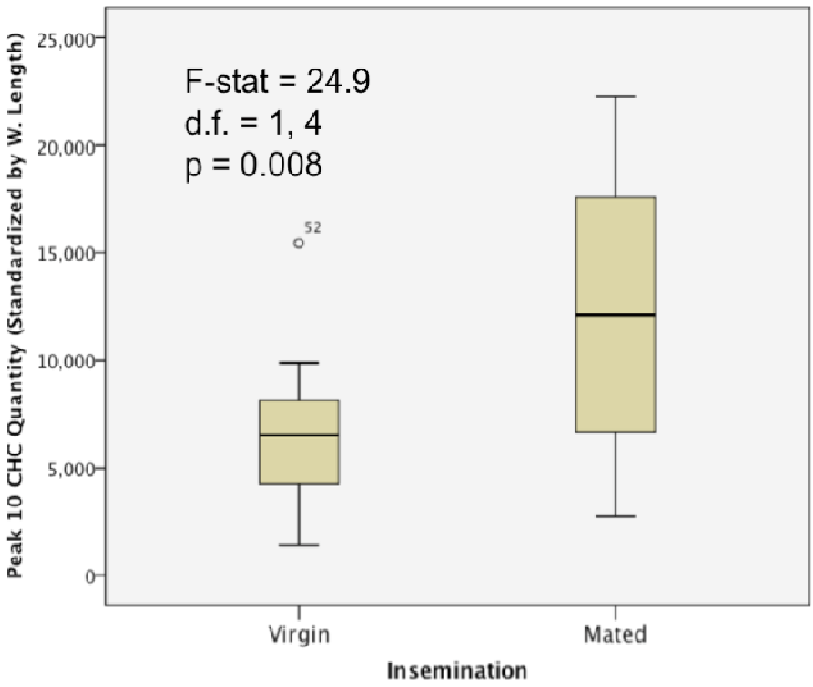

Figure 13. Effect of Insemination on Mean Peak 10 CHC Quantity.